Packed-bed bioreactor synthesis of feruloylated monoacyl- and diacylglycerols: clean production of a "green" sunscreen†‡

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A biocatalytic process for covalent incorporation of ferulic acid onto the glycerol backbone of vegetable oil proceeds efficiently, although rather slowly, with *Candida antarctica* lipase B in a packed-bed reactor. The bioreactor shows considerable long-term stability. Product yield is influenced by the water content of the fluid phase and enzyme support. The enzyme support modulates substrate concentrations through adsorption and subsequent release of reactants over the course of the reaction. The resulting product has excellent UVA/UVB absorbing properties, making it a potential substitute for conventional petroleum-based sunscreen active agents.

Introduction

Heightened awareness of the skin damaging effects of ultraviolet (UV) radiation by the public has lead to robust growth in sun and skin care personal product markets. While UV-B (290–320 nm) radiation is principally responsible for sunburn (erythemogenic effect), UV-A (320–400 nm) radiation promotes photo-damage and aging of the skin. A preponderance of sunscreens (80%) in U.S. and Western Europe markets rely on organic chemicals to provide UV protection.[1] The active ingredients most commonly employed in the U.S. are octyl methoxycinnamate (OMC), padimate-O (*N*,*N*-dimethyl-*p*-aminobenzoic acid octyl ester), and oxybenzone (2-hydroxy-3-methoxybenzophenone). In Western Europe, 4-methylbenzyldiene camphor (4-MBC) is also a prevalent sunscreen active ingredient. Use of these chemicals is quite high, as an active ingredient may constitute up to 25% by weight or volume of the sunscreen formulation.[2]

Recently, concerns have been raised about the potential adverse health and ecological effects of the commonly used sunscreen active ingredients.[3,4] The estrogenic activity of OMC and 4-MBC have been documented *in vitro* and *in vivo* with mice.[5] Although the ability of these chemicals to disrupt endocrine activity in humans has yet to be established, these findings serve to legitimize in the public's mind a desire to avoid "synthetic" chemicals and to prefer "natural" ingredients in their personal care products.[6] Current sunscreen active ingredients may also pose an ecological threat. As with other pharmaceuticals, these chemicals tend to be bioaccumulative and biopersistent.[7] Lakes frequented by sunbathers may have aquatic life impacted from the presence of relatively high levels of sunscreen active ingredients. These concerns indicate that a benign alternative to conventional UV active ingredients would be well accepted in the marketplace.

A sunscreen active ingredient can be derived from two natural plant components, ferulic acid and triglycerides.[8] Ferulic acid is a phenolic compound (a member of the cinnamic acid family) found in most higher plants. It is generally present in nature as esters with other plant components, such as the hemicelluloses and lignin fractions of the plant cell wall, as well as in suberin and cutin waxy surfaces of leaves and other plant parts. Ferulic acid is also found esterified to phytosterols present in grain products such as rice bran. As such, ferulic acid is a common component of the human diet. It is thus not expected to pose a threat to human health nor to the environment.

Transesterification of ferulic acid ethyl ester with vegetable oil produces a mixture of feruloylated monoacyl- and diacylglycerols that have a strong UVA/B absorbance as well as water resistance characteristics.[8,9] The reaction is catalyzed by immobilized *Candida antarctica* lipase B. Previous studies have shown the reaction to proceed efficiently, albeit rather slowly (144 h to reach equilibrium), in stirred batches of the enzyme.[9] While it was demonstrated that the immobilized enzyme could be used several times, it is likely that on a commercial production scale degradation and loss of the enzyme from its support would be severe with stirred batch reactors. In the present work, the use of the immobilized enzyme in packed beds is examined, anticipating that the mild operating conditions would greatly extend the service life of the catalyst. The value of using packed-bed bioreactors for the transformation of vegetable oils has been recently demonstrated.[10–13] Packed-bed processing should also allow a readily scalable approach to producing commercial quantities of the product. In addition, issues related to the pre- and post-production of product are addressed, demonstrating a synthesis

Green Context

The increasing awareness of the dangers of exposure to the sun is making worse the impact of sunscreens on the environment, especially aquatic systems. This paper reports on a clean alternative, based on the combination of naturally occurring phenolic compounds onto glycerol. The synthesis of the product is analysed in terms of the principles of green chemistry, and is seen to have a high degree of atom economy, as well as being (probably) very biodegradable.

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† Electronic supplementary information (ESI) available: schematics of the packed-bed reactor and the CO_2 fractionation system, HPLC chromatographic analysis of SoyScreen™. See http://www.rsc.org/suppdata/gc/b3/b302384b/

‡ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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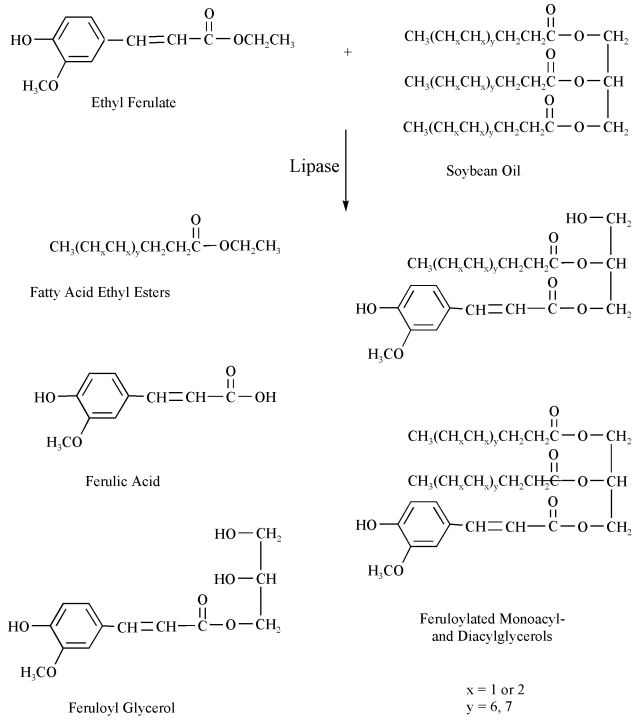
of a plant-derived, environmentally benign sunscreen that closely adheres to the tenets of green chemistry.

Results and discussion

Reaction analysis

Transesterification of soybean oil (SBO) with ethyl ferulate (EF) by immobilized lipase *Candida antarctica* lipase B (Novozym 435) produces a multitude of long-UV-absorbing feruloylated monoacylglycerols (FMG) and feruloylated diacylglycerols (FDG), collectively referred to as SoyScreen™ (Scheme 1). Equal proportions of the FMG and FDG are



Scheme 1 Lipase reaction substrates and principal products. Feruloyl group substitution at the glycerol *sn*-3 position is shown for simplicity as the regioselectivity of the reaction is unknown.

produced. Additional UV-absorbing products formed in relatively small quantities are ferulic acid (FA), resulting from EF hydrolysis, and feruloyl glycerol (FG). The principal by-products from the reaction are fatty acid ethyl esters (FAEE). With an initial 1 : 1 molar ratio of EF to triglyceride, approximately 50% of the starting materials should convert to products at equilibrium, at which point the concentration of unreacted EF will equal that of the FAEE, so no further productive reaction will ensue. Generation of FA in this reaction is essentially irreversible as the enzyme is unable to appreciably esterify this substrate.[9] FG remains a reactive species and for the purposes of this study is considered a constituent of the SoyScreen™ product. Diferuloyl-substituted species have not been detected.[9]

The extent of conversion of EF and SBO to SoyScreen™ was determined most accurately by measuring residual EF and FA by high performance liquid chromatography (HPLC) rather than following the appearance of FMG and FDG. The latter approach overestimates the progress of the reaction. An additional correction was applied to the measurement of EF and FA in a sample by adjusting for small variations in the total ferulates (TF) concentration (*i.e.*, all feruloyl species, including EF and FA, monitored by UV spectroscopy at 325 nm), due to adsorption and desorption of EF to the enzyme support over the course of the reaction (see below). Thus the extent of SoyScreen™ production (θ) was calculated as

$$\theta = (1 - \frac{[EF] + [FA]}{[TF]})$$

Bioreactor kinetics

The performance of a packed bed of Novozym 435 in the conversion of EF and SBO to SoyScreen™ was examined for the time required for the reaction to reach completion (Fig. 1).

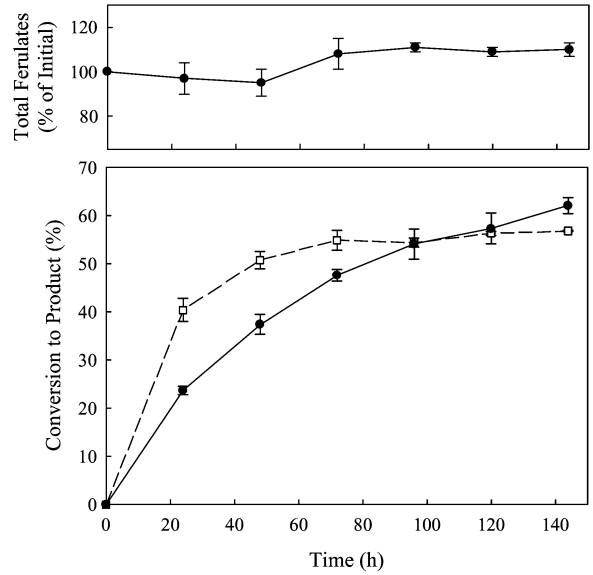


Fig. 1 Influence of bioreactor residence time on the conversion of EF to SoyScreen™ (lower panel) and the total ferulate concentration (upper panel). Data for the conversion to SoyScreen™ are the average of three trials made with fresh enzyme in the packed-bed bioreactor (●, solid line). Reaction results with immobilized enzyme (150 mg mL^{-1} of reactants) in stirred vials are the average of three separate trials (□, dashed line). Total ferulate concentration data are the average of four consecutive runs made with the same enzyme bed in the bioreactor.

For a freshly packed reactor the extent of reaction approached completion after approximately 144 h of operation. In contrast, the transesterification of triolein with caprylic acid ethyl ester by Novozym 435 reached equilibrium after 24 h at 45 °C.[14] Thus the kinetics of transesterification by the enzyme of SBO with EF are slow in comparison to those with more preferred acyl donors (long-chain fatty acid esters rather than cinnamic acid esters). The highest yield of SoyScreen™ observed after 144 h was 64%, which is somewhat greater than expected based on a simple equilibrium model of transesterification. This observation may indicate that the feruloyl substituted acylglycerols are slightly more thermodynamically stable than their corresponding diacyl- and triacylglycerols.

Stirred batches of immobilized enzyme and the SBO–EF reactants produced results similar to that of the packed-bed bioreactor (Fig. 1). Product yield was higher initially for stirred batches, but ultimately lower than with the packed-bed approach. Fracture of the enzyme support was evident in the stirred-batch reactions. The rapid initial kinetics of the reaction in stirred batches, followed by a relatively slower progression compared to the packed-bed reactor, may arise from greater surface reaction from the pulverized enzyme support and accompanying attrition of active enzyme by abrasion.

Continuous operation of the bioreactor over extended periods showed that multiple batches of SoyScreen™ could be prepared from a single charge of enzyme. Product yield dropped below 60% after the first two weeks of operation, then fluctuated

between 52 and 58% for the subsequent four weeks (data not shown). The apparent minor loss of enzyme activity after the first few batches cannot be attributed to occlusion of the support beads or channeling within the reactor because the measured included volume of the reactor bed did not change over this period of operation. Therefore, the small change in reactor performance with time most likely was due to thermal denaturation of the enzyme or changes in water activity (see below).

Influence of water

The amount of FA produced in the reaction was dependent on the water content of the reaction medium. Without a drying column, the FA content of the reaction medium slowly rose to represent 5–7% of the total ferulates by 144 h, while with a drying column the FA concentration was less than 2% (Fig. 2).

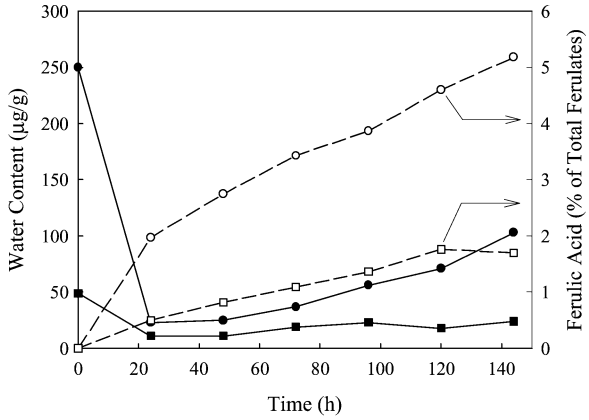


Fig. 2 Changes in the water content (solid lines; left axis) and ferulic acid concentration (dashed lines; right axis) of the fluid phase of the bioreactor during SoyScreen™ synthesis. The initial water content of the SBO was either left high (○, ●) or was lowered by drying with molecular sieves (□, ■), in which case a pre-column of molecular sieves was included in the flow stream of the reactor.

Without a drying column in line before the bioreactor, the water concentration in the reactor effluent still dropped dramatically to less than 10% of its initial concentration within the first 24 h of circulation, indicating that the enzyme support was adsorbing water. The pattern of water concentration in the SoyScreen™ over the course of the reaction suggests that the support acts as both a sink and source for water as the water solubility characteristics of the fluid phase change with time (*i.e.*, from a mixture of SBO and EF initially to the complex mixture of components comprising SoyScreen™ after 144 h of reaction). The partitioning of water between the enzyme support and fluid phase in packed-bed reactors has been noted by others.[15]

Water is not a product of the transesterification process and thus does not originate from the reaction itself. The enzyme support as supplied contains 1% (w/w) water according to the manufacturer, thus providing approximately 340 mg of water to the system. Each charge of reactants (EF and SBO) at the start of a transesterification run provides another 60 mg of water (240 mL containing 0.25 mg mL^{-1} of water), with the rinse SBO used to displace the product from the column after the completion of the reaction providing an additional 30 mg. With this protocol, sufficient water was introduced with each charge and discharge of the reactor to account for the ferulic acid produced. Use of an external column with molecular sieves helped minimize ferulic acid production by limiting the available water to that which came with the enzyme and its support.

Most enzymes need a critical amount of water to retain enzymatic activity in non-aqueous media, typically displaying a "bell-shaped" response to water activity.[16,17] This rule applies to lipases as well, with the apparent exception of *Candida antarctica* lipase B. It is generally observed that this lipase expresses its highest catalytic rates at very low water activities.[18–22] Thus it was surprising to see in our reaction lower product yield at very low water content in the medium (Fig. 3).

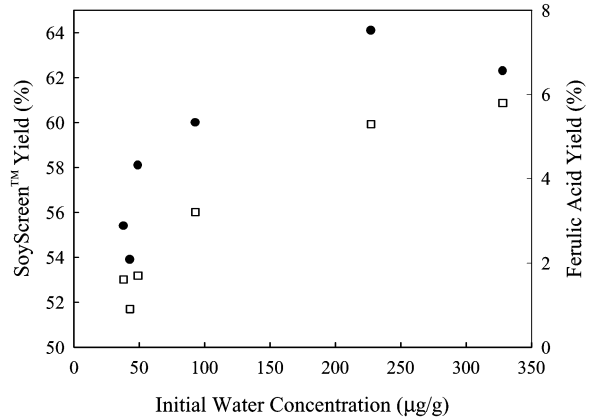


Fig. 3 Relationship between initial reactor water content and yield of SoyScreen™ (●; left axis) and ferulic acid (□; right axis) after 144 h. Initial water content was adjusted by drying the SBO with molecular sieves or by adding water to the EF–SBO mixture at the beginning of the reaction.

The large volumes of reactants employed and the high ratio of enzyme support to fluid phase in the present work made controlling water activity by conventional means such as hydrated salt complexes not feasible. Therefore, to study the influence of water on the reaction, only the initial water content of the fluid phase was adjusted. Increasing water concentration raised enzyme activity, raising product yield, but escalating as well the rate of EF hydrolysis (Fig. 3). This indicates that optimization of the reaction will require consideration of the concurrent trends of increasing SoyScreen™ yield (to a limit) and increasing ferulic acid by-product production with greater water activity.

Evaluation of EF adsorption to support

The concentration of total ferulates in the recirculated eluate from the reactor varied in a consistent manner of being slightly lower initially than the applied EF concentration, but then slowly rising over the 144 h reaction period to 10% above the applied EF concentration (Fig. 1). The variation in total ferulates with time can be attributed to the adsorption/desorption of EF to the enzyme's acrylic support. The adsorption properties of Novozym 435 were investigated using heat-inactivated material. There was substantial adsorption of EF to the enzyme support with SBO as the solvent. Over the EF concentration range relevant to this study, 0.2 to 0.9 M, the extent of EF adsorption increased linearly from 70 to 270 mg EF g $support^{-1}$ (data not shown). To minimize the effects of EF adsorption on the transesterification reaction kinetics, the resin was pre-equilibrated with a volume of EF in SBO (*i.e.*, a volume, equal to that which was recirculated, was passed through the column and discarded). The reaction products, FMG and FDG, showed little affinity for the enzyme support (data not shown). Therefore, only EF adsorption and desorption from the enzyme support contributed to the total ferulate concentration fluctuations during the course of the transesterification reaction.

Dossat and colleagues[12] found that glycerol generated during the transesterification of sunflower oil with 1-butanol in a packed-bed bioreactor of Lipozyme accumulated on the enzyme support and diminished enzyme activity. The amount of glycerol generated in the SoyScreen™ reaction was not

evaluated. However, adsorption of EF to the support may have a similar impact on the reactivity of Novozym 435, acting as a physical barrier to diglyceride and triglyceride reactants reaching the enzyme.

Post-reactor processing

Unreacted EF could be separated from SoyScreen™ at the end of the bioreactor stage. Liquid CO_2 (25 °C, 8.6 MPa) percolated through SoyScreen™ extracted EF, FAEE and FA. The FA formed a white precipitate in the extract. Passage of 3000 L (STP) of CO_2 lowered EF concentrations in the product to trace levels. No SoyScreen™ products or triglycerides were removed by liquid CO_2. Attempts to separate EF from the FAEE fraction using liquid and supercritical CO_2 were unsuccessful.

As with liquid CO_2 extraction, EF, FA and FAEE were readily separated from the SoyScreen™ products by low temperature molecular distillation (120 °C). FA crystallized from the distillate on cooling below room temperature. Complete removal of EF from the residue (SoyScreen™) fraction would require a second pass through the apparatus. This is a limitation of the bench-scale unit employed and would not be expected to be necessary at higher production volumes.

FAEE and EF recovered from either liquid CO_2 extraction or high vacuum distillation were converted to SoyScreen™ by adding glycerol or diacetin (glycerol diacetate) and Novozym 435 in a batch reactor. With the glycerol or diacetin concentration approximately equimolar to the EF, conversion to SoyScreen™ proceeded to about 50% after 144 h. Greater conversion would be expected if the competing transesterification products, either ethanol or ethyl acetate, were driven off under reduced pressure during the reaction, although this was not attempted.

How green is SoyScreen™?

Both the product and the processes to produce SoyScreen™ adhere closely to the principles of green chemistry.[23] SoyScreen™ is likely to be biodegradable and to have negligible toxicity, although this has yet to be verified. The raw materials for SoyScreen™, FA and SBO, are from annually renewable resources. A preliminary assessment indicates that FA can be isolated from phytosterol fractions (these sterols have commercial value as well) more economically than by *de novo* chemical synthesis.[24,25] Esterification of FA in ethanol to make EF can be accomplished using recyclable solid acid catalysts. Ethanol is the only reaction component that may not be practically conserved in the process, so SoyScreen™ synthesis has a high degree of atom economy. No solvents, separating agents (other than CO_2 perhaps), or intermediary protecting groups are needed. Processing substrates and products with liquid CO_2 is particularly advantageous because solutes are readily recovered by partial depressurization, which also lessens the energy costs associated with its use. The distillation steps for EF synthesis and SoyScreen™ fractionation are the most energy intensive steps, yet their requirements are quite modest. The biocatalyst shows long term stability in a packed-bed reactor. Future work will focus on improving catalyst turnover rates to shorten synthesis times.

Experimental

Reagents

Novozym 435 was obtained from Novo Nordisk BioChem North America (now Novozymes North America, Franklinton, NC). EF (ethyl 4-hydroxy-3-methoxycinnamate) was purchased from Senn Chemicals USA (San Diego, CA). Colored contaminants were removed from the EF by either passage through alumina (60–325 mesh, Acid Brockman Activity I) with the EF dissolved in acetone, or by liquid CO_2 extraction (see below). SBO was obtained from a local grocery store. Other reagents were from Sigma-Aldrich and Fisher Scientific. Glycerol was spectroscopic grade (< 0.1% w/w water). Diacetin was technical grade.

Packed-bed bioreactor

Novozym 435 (34 g) was solvated in SBO under reduced pressure for 30 min, then transferred to a jacketed chromatography column (2.5 × 30 cm, 147 mL nominal internal volume). Using β-carotene as a marker, the bed included volume was estimated to be 85 mL. The enzyme bed was conditioned overnight by recirculating about 340 mL of SBO at 2 mL min^{-1}. The reactor was maintained at 60 °C using a circulating bath. Reactants (EF and SBO) were fed into the top of the reactor using a peristaltic pump at 2 mL min^{-1}. Reactor effluent was collected in a small reservoir (30 mL), which was kept under a slow stream of N_2 with the contents magnetically stirred, and recirculated back into the packed-bed bioreactor. The reaction mixture was prepared by combining 40 g of EF with 160 g of SBO at 60 °C. While retaining 25 mL of this solution for the reservoir, the reaction mixture was passed onto the column, discarding the displaced SBO to waste, then directing the reactants back to the reservoir once the reactor was entirely loaded. (See the supplemental materials for a schematic of the apparatus†). A drying bed of 3A molecular sieves (8 g), when used, was placed between the pump and the reactor column.

Samples (0.5 mL) were collected daily from the column effluent for analysis by HPLC and UV spectroscopy (325 nm). Sample water content was determined using coulometric Karl Fischer analysis with 70 : 30 (v/v) Hydranal AG–H/chloroform as the analyte.

HPLC analysis

Samples from the bioreactor were analyzed by HPLC largely following previously published procedures.[9] A Thermo Separation Products (San Jose, CA) HPLC was equipped with a UVB-visible detector and a Prodigy C8 column (Phenomenex, Torrance, CA). For the separation of various feruloyled lipids, the column was developed isocratically at 1.5 mL min^{-1} with 40 : 60 (v/v) acetone (containing 1% glacial acetic acid)–acetonitrile. Samples were prepared by 200-fold dilution into acetone. The eluate was monitored at 360 nm. For the quantitation of FA, FG, and EF, a water–methanol gradient elution regime was employed, with detection at 325 nm and with the acetone-diluted samples further diluted 20-fold with methanol.[9] (See supplemental materials for example chromatograms†). Detector response (325 nm) was calibrated with FA and EF, using low concentrations of EF to establish a calibration curve for FG. Responses were linear for all three species in the range employed in this study (100 to 250 μM for EF, 2 to 20 μM for FA and FG). The sample injection volume was 10 μL for both modes of analysis.

Measurement of EF adsorption

For the study of EF adsorption to the support resin, Novozym 435 was autoclaved to inactivate the enzyme. SBO (1 mL) containing 0.25 to 1.0 M EF was equilibrated with 150 mg of autoclaved Novozym 435 in stirred vials at 60 °C for 24 h. The equilibrated concentration of EF was determined by UV spectroscopy (325 nm). The amount of adsorbed EF was taken

as the difference between the initial and final EF concentrations.

Carbon dioxide extraction

Liquid CO_2 fractionations were performed in a column packed with protruded stainless-steel packing (0.41 cm Pro-Pak, Scientific Development Co., State College, PA), which provided a 94% void volume. The column included a precooling section and four separate zones, each having an internal diameter of 1.43 cm and a height of 63.3 cm (see supplemental materials for a schematic diagram of the CO_2 fractionation system†). The column had a total height of 253.2 cm and an internal volume of 412 mL.

For liquid CO_2 extraction, all zones were cooled to 25 °C by silicone tubing attached to a refrigerated circulating bath. Heating mantles enclosed each zone and were independently controlled to heat the column to the desired temperature during the post-extraction cleanup. The temperature was recorded by Type-J thermocouples attached to the column wall. Two thermocouples were inserted into the top and bottom of the column to monitor the internal column temperature.

Sample (120 mL of unextracted SoyScreen™) was introduced into the column above the first zone by a liquid metering pump (Model MS-188, Haskel Inc., Burbank, CA) connected to a stroke counter controller. Welding-grade carbon dioxide (Airgas Inc., Radnor, PA) was introduced from a commercial cylinder through a filter containing alumina C to a booster pump (Model AG-30, Haskel Inc., Burbank, CA). The column was pressurized to 8.6 MPa and equilibrated for 0.5 h before the outlet valve was opened to begin the extraction. CO_2 was passed upward through the column and sample. Solute-loaded liquid CO_2 exited the column as expanded gas at a flow rate of 3–5 L min^{-1} (STP) across a micrometering valve allowing the extract (EF, FA and FAEE) to be collected in a flask. Finally, the gas stream passed through a dry test meter (Singer Model DTM-115, American Metering Division, Philadelphia, PA) to measure the total gas volume and was then vented to the atmosphere. Purified SoyScreen™ was then collected from the bottom of the second zone through a micrometering valve as the column was depressurized.

After each extraction, the column was cleaned to prevent contaminants from being carried over to subsequent runs. The refrigerated circulating bath was turned off and the column was heated to 80 °C and pressurized to 58.6 MPa with supercritical CO_2. One-thousand L (STP) of CO_2 were run through the column at 5 L min^{-1} for cleanup.

Molecular distillation

SoyScreen™ fractionation also was performed using high vacuum (short-path) distillation (Myers Vacuum, Inc., Ittanning, PA). The centrifugal rotor temperature was 120 °C and the condensing surface was held at 40 °C with the chamber operating at 16 mTorr.

Acknowledgements

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References

1 M. S. Reisch, *Chem. Eng. News*, 2001, **79**(Dec. 3), 25–29.
2 M. E. Griffin, T. D. Bourget and N. J. Lowe, *Sunscreens. Development, Evaluation, and Regulatory Aspects*, ed. N. J. Lowe, N. A. Shaath and M. A. Pathak, Marcel Dekker, New York, 1997, pp. 499–512.
3 J. J. P. Morton, *J. Toxicol. Cutaneous Ocul. Toxicol.*, 1992, **11**, 239–247.
4 M. Schlumpf, B. Cotton, M. Conscience, V. Haller, B. Steinmann and W. Lichtensteiger, *Environ. Health Perspect.*, 2001, **109**, 239–244.
5 G. J. Nohynek and H. Schaefer, *Regul. Toxicol. Pharmacol.*, 2001, **33**, 285–299.
6 M. S. Reisch, *Chem. Eng. News*, 2001, **79**(Apr. 16), 23–28.
7 C. G. Daughton and T. A. Ternes, *Environ. Health Perspect.*, 1999, **107**(suppl. 6), 907–938.
8 D. L. Compton and J. A. Laszlo, *US Pat.*, 6346236, 2002.
9 D. L. Compton, J. A. Laszlo and M. A. Berhow, *J. Am. Oil Chem. Soc.*, 2000, **77**, 513–519.
10 F. X. Malcata, H. R. Reyes, H. G. Garcia and C. G. Hill Jr., *J. Am. Oil Chem. Soc.*, 1990, **67**, 890–910.
11 Y. Watanabe, Y. Shimada, A. Sugihara and Y. Tominaga, *J. Am. Oil Chem. Soc.*, 2001, **78**, 703–707.
12 V. Dossat, D. Combes and A. Marty, *J. Biotechnol.*, 2002, **97**, 117–124.
13 L. B. Fomuso and C. C. Akoh, *Food Res. Int.*, 2002, **35**, 15–21.
14 K.-H. Huang and C. C. Akoh, *J. Am. Oil Chem. Soc.*, 1996, **73**, 245–250.
15 S. Colombié, R. J. Tweddell, J.-S. Condoret and A. Marty, *Biotechnol. Bioeng.*, 1998, **60**, 362–368.
16 L. Kvittingen, *Tetrahedron*, 1994, **50**, 8253–8274.
17 G. Koller, M. J. Aris, Z. Ujang and A. M. Vaidya, *Biocatal. Biotransform.*, 2001, **19**, 37–49.
18 S. Barreiros, N. Fontes, M. Conceicão Almeida, R. Ruivo and T. Corrêa de Sampaio, *4th International Symposium on Supercritical Fluids*, May 11–14, Sendai, Japan, 1997, pp. 111–113.
19 M.-P. Bousquet-Dubouch, M. Graber, N. Sousa, S. Lamare and M.-D. Legoy, *Biochim. Biophys. Acta*, 2001, **1550**, 90–99.
20 F. Chamouleau, D. Coulon, M. Girardin and M. Ghoul, *J. Mol. Catal. B: Enzym.*, 2001, **11**, 949–954.
21 J.-H. Xu, Y. Kato and Y. Asano, *Biotechnol. Bioeng.*, 2001, **73**, 493–499.
22 Y. Li and D. G. Rethwisch, *Biotechnol. Bioeng.*, 2002, **79**, 15–22.
23 P. T. Anastas and M. M. Kirchhoff, *Acc. Chem. Res.*, 2002, **35**, 686–694.
24 H. Taniguchi, A. Hosoda, T. Tsuno, Y. Maruta and E. Nomura, *Anticancer Res.*, 1999, **19**, 3757–3762.
25 H. Taniguchi, E. Nomura, T. Tsuno and S. Minami, *US Pat.*, 5908615, 1999.